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STABILIZING METHOD OF CHOLESTEROL OXIDASE

(Koresuteroru Okishidaze no Anteika Ho)

Naoka HARADA, Yoshiaki NISHIYA, Kazumi YAMAMOTO

Shinichi TEJIMA and Shigenori AISUI

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Inventor(s)	:	Naoka HARADA
		Yoshiaki NISHIYA
		Kazumi YAMAMOTO
		Shinichi TEJIMA
		Shigenori AISUI
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SPECIFICATION

(54) Title of the Invention

Stabilizing Method of Cholesterol Oxidase

[Claims]

[Claim 1] A stabilizing method of cholesterol oxidase, which is characterized by adding at least one compound selected from a group composed of (1) bovine serum albumin and (2) sugars and amino acids into a solution containing cholesterol oxidase.

[Claim 2] The stabilizing method of cholesterol oxidase described in Claim 1, in which at least one compound selected from a group composed of (1) bovine serum albumin and (2) sugars and amino acids is added into a solution containing cholesterol oxidase and the mixture is further freeze-dried.

[Claim 3] The stabilizing method of cholesterol oxidase described in Claim 1, in which the sugars are at least one compound selected from a group composed of sucrose, glucose, trehalose, lactose, sodium gluconate and mannitol.

[Claim 4] The stabilizing method of cholesterol oxidase described in Claim 1, in which the amino acids are at least one

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[1]Numbers in the margin indicate pagination in the foreign text.

compound selected from a group composed of sodium glutamate, lysine and glycine.

[Claim 5] The stabilizing method of cholesterol oxidase described in Claim 1, in which the sugars are at least one compound selected from a group composed of sucrose, glucose, trehalose, lactose, sodium gluconate and mannitol and the amino acids are at least one compound selected from a group composed of sodium glutamate, lysine and glycine.

[Claim 6] The stabilizing method of cholesterol oxidase described in Claim 1, in which 0.1 ~ 20% of bovine serum albumin, 0.1 ~ 20% of sugars and 0.1 ~ 20% of amino acids are added to the cholesterol oxidase solution.

[Claim 7] A cholesterol oxidase formulation, which is characterized by containing cholesterol oxidase and at least one compound selected from a group composed of (1) bovine serum albumin and (2) sugars and amino acids.

[Claim 8] The cholesterol oxidase formulation described in Claim 7, which is a solid formulation.

[Claim 9] The cholesterol oxidase formulation described in Claim 7, in which the sugars are at least one compound selected from a group composed of sucrose, glucose, trehalose, lactose, sodium gluconate and mannitol.

[Claim 10] The cholesterol oxidase formulation described in Claim 7, in which the amino acids are at least one compound

selected from a group composed of sodium glutamate, lysine and glycine.

[Claim 11] The cholesterol oxidase formulation described in Claim 7, in which the sugars are at least one compound selected from a group composed of sucrose, glucose, trehalose, lactose, sodium gluconate and mannitol and the amino acids are at least one compound selected from a group composed of sodium glutamate, lysine and glycine.

[Claim 12] A reagent kit for cholesterol oxidase measurement, which comprises a cholesterol esterase formulation, a cholesterol oxidase formulation containing cholesterol oxidase and at least one compound selected from a group composed of (1) bovine serum albumin and (2) sugars and amino acids, a peroxidase, 4-aminoantipyrin, an aniline derivative or a phenol derivative and a buffer.

[Claim 13] A stabilizing method of cholesterol oxidase, which is characterized by adding lysine into a solution containing cholesterol oxidase.

[Claim 14] The stabilizing method of cholesterol oxidase described in Claim 13, in which lysine is added into a solution containing cholesterol oxidase and the mixture is further freeze-dried.

[Claim 15] The stabilizing method of cholesterol oxidase described in Claim 13, in which lysine and bovine serum albumin

are added into a solution containing cholesterol oxidase and the mixture is further freeze-dried.

[Claim 16] The stabilizing method of cholesterol oxidase described in Claim 13, in which 0.1 ~ 20% of lysine is added into the solution containing cholesterol oxidase.

[Claim 17] A cholesterol oxidase formulation, which is characterized by containing cholesterol oxidase and lysine.

[Claim 18] The cholesterol oxidase formulation described in Claim 17, which contains cholesterol oxidase, lysine and bovine serum albumin.

[Claim 19] The cholesterol oxidase formulation described in Claim 17, which is a solid formulation.

[Claim 20] A reagent kit for cholesterol oxidase measurement, which comprises a cholesterol esterase formulation, a cholesterol oxidase formulation containing cholesterol oxidase and lysine, a peroxidase, 4-aminoantipyrin, an aniline derivative or a phenol derivative and a buffer.

[Detailed Description of the Invention]

[0001]

[Field of Industrial Application] The present invention relates to a stabilizing method of cholesterol oxidase, and particularly to a stabilizing method of cholesterol oxidase that is clinically used in measurement of cholesterol oxidase in body

fluid becoming an indicator for diagnosis of endocrinoses and dysbolism.

[0002]

[Prior Art] Cholesterol oxidase is an enzyme that is clinically used in measurement of cholesterol oxidase in body fluid becoming an indicator for diagnosis of endocrinoses and dysbolism. As stabilizing methods of cholesterol oxidase, the addition

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of alkali metal salts, alkali-earth metal salts (Japan Tokkyo 57-13272) and the addition of bovine serum albumin, dextran, polyethylene glycol (Japan Kokai 06-62846) have been known before. However, there were problems of insufficient stabilization, lowering of activity of enzyme in a preservation period and formation of turbidity, etc. in the addition of these substances, particularly, no description of turbidity has been found and a radical solution has not been make so far.

[0003]

[Problem to Be Solved by the invention] The present invention relates to a stabilizing method of cholesterol oxidase, and its purpose consists in providing a stable enzyme that does not lower the enzymatic activity in long period preservation. The stabilization mentioned here means two points, i. e., the retention of enzymatic activity and the inhibition of turbidity formation from the enzyme caused by denaturation.

[0004]

[Means for Solving the Problem]

The present invention is a stabilizing method of cholesterol oxidase, which is characterized by adding at least one compound selected from a group composed of (1) bovine serum albumin and (2) sugars and amino acids into a solution containing cholesterol oxidase.

[0005]

Moreover, the present invention is a cholesterol oxidase formulation, which is characterized by containing cholesterol oxidase and at least one compound selected from a group composed of (1) bovine serum albumin and (2) sugars and amino acids.

[0006]

Furthermore, the present invention is a reagent kit for cholesterol oxidase measurement, which comprises a cholesterol esterase formulation, a cholesterol oxidase formulation containing cholesterol oxidase and at least one compound selected from a group composed of (1) bovine serum albumin and (2) sugars and amino acids, a peroxidase, 4-aminoantipyrin, an aniline derivative or a phenol derivative and a buffer.

[0007] The present invention is a stabilizing method of cholesterol oxidase, which is characterized by adding lysine into a solution containing cholesterol oxidase.

[0008] Moreover, the present invention is a cholesterol oxidase formulation, which is characterized by containing cholesterol oxidase and lysine.

[0009] Furthermore, the present invention is a reagent kit for cholesterol oxidase measurement, which comprises a cholesterol esterase formulation, a cholesterol oxidase formulation containing cholesterol oxidase and lysine, a peroxidase, 4-aminoantipyrin, an aniline derivative or a phenol derivative and a buffer.

[0010] Cholesterol oxidases of any origins may be used as those used in the present invention, for example, cholesterol oxidases produced by micro-organisms such as *Streptomyces*, *Corynebacterium*, *Brevibacterium*, *Norcadia*, etc. are given. Cultures containing cholesterol oxidases obtained by culturing these microorganism, crude enzymes obtained by extracting said cultures according to ordinary methods or purified enzymes obtained by purifying said crude enzymes according to ordinary methods are preferably used. Enzymes obtained by modifying these enzymes by gene engineering or chemically, physically are also included.

[0011] The invented method for formulating stabilizers is not specially restricted. For example, a method of formulating stabilizers in a buffer containing cholesterol oxidase, a method of formulating cholesterol oxidase in a buffer containing stabilizers, a method of adding cholesterol oxidase and stabilizers

into a buffer at the same time, etc. are given. It is preferable that an enzyme solution formulated with stabilizers is freeze-dried in the present invention. Conditions of freeze-drying are not specially restricted.

[0012] Any buffers such as phosphoric acid buffer, good buffer and other buffers used in biochemistry may be used as those used in the present invention, the pH is 4 ~ 10, desirably pH 6.0 ~ 8.0.

[0013] The bovine serum albumin concentration used in the present invention is 0.1 ~ 20% to a cholesterol oxidase solution. However, as shown in the following Table 1, the residual activity is only 88% with bovine serum albumin alone in preservation at 37°C for one week, thus a sufficient stabilization effect was not obtained. Moreover, the formation of turbidity was also striking.

[0014] As sugars used in the present invention, monosaccharides such as glucose, galactose, xylose, fructose, etc., disaccharides such as lactose, maltose, etc., polysaccharides such as trehalose, sucrose, gluconic acid are given, particularly sucrose, glucose, trehalose, lactose, sodium gluconate and mannitol are preferable. The concentration is 0.1 ~ 20 to solution. If the cholesterol oxidase was preserved with sugars alone at 37°C for one week, as shown in the following Table 1, a sufficient stabilization effect was not obtained and the formation of

turbidity was also striking. The sugars may also be used by combining one, two or more of them.

[0015] As amino acids or their salts used in the present invention, hydrophilic amino acids such as glycine, glutamic acid, lysine, etc. or salts such as their sodium, potassium, ammonium salts, acetates or hydrochlorides, etc. are given, glycine aminoacetate, lysine hydrochloride are more preferable. The concentration is 0.1 ~ 20 to cholesterol oxidase solution. If cholesterol oxidase was preserved at 37°C for one week with amino acids (except for lysine) alone, as shown in the following Table 1, a sufficient stabilization effect was not obtained and the formation of turbidity was also striking. The amino acids may also be used by combining one, two or more of them.

[0016] A preferred embodiment of the present invention is a stabilizing method of cholesterol oxidase in which at least one compound selected from a group composed of (1) bovine serum albumin and (2) sugars and amino acids is added into a solution containing cholesterol oxidase and the mixture is further freeze dried.

[0017] Another preferred embodiment of the present invention is a stabilizing method of cholesterol oxidase in which lysine is added into a solution containing cholesterol oxidase and the mixture is further freeze dried.

[0018] Still another preferred embodiment of the present invention is a cholesterol oxidase solid formulation containing contains cholesterol oxidase and at least one compound selected from a group composed of (1) bovine serum albumin and (2) sugars and amino acids.

[0019] Still further another preferred embodiment of the present invention is a cholesterol oxidase solid formulation containing cholesterol oxidase and lysine.

[0020] Hydrogen peroxide formed by the action of cholesterol oxidase is measureded by various well-known methods with the reagent kit for cholesterol oxidase measurement of the present invention. A method wherein a chromogen is oxidized in the presence of peroxidase, reacted with a coupler such as 4-aminoantipyrin to form a colorant and then quantified by colorimetry is given as the most commonly-used method. As chromogens used in this method, phenol, phenol derivatives such as 2-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, etc. or aniline, aniline derivatives such as N,N-dimethylaniline, N,N-diethylaniline, N,N-diethyl-m-toluidine, N,N-diethyl-m-anisidine, N-ethyl-N-(3-methylphenyl)-N'-acetylethylenediamine, N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-anisidine, N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine, N-ethyl-N-sulfopropyl-m-toluidine, N-ethyl-N-sulfopropyl-m-anisidine, etc. are given. Reagents for determining hydrogen peroxide are not restricted to the above reagents.

[0021]

[Actual Examples] The present invention is described in detail by actual examples.

Actual Example 1

17.2 mg of a bovine serum albumin (abbreviated as BSA hereafter, made by Organotechnic Co.) and 14.7 mg of one selected from a group composed of sucrose, glucose, trehalose, lactose, sodium gluconate, mannitol, sodium glutaminate and glycine were mixed with 1 mL of a cholesterol oxidase solution (2,000 U/mL), respectively, and the mixtures were freeze-dried to obtain 50 mg of freeze dry powders. Similarly, freeze dry powders were obtained by adding or not adding each additive alone.

[0022] The freeze dry powders of cholesterol oxidase added with bovine serum albumin and one of sugars or amino acids and obtained by the above preparation, a freeze dry powder added with 17.2 mg of BSA alone into a solution of cholesterol oxidase, a freeze dry powder added with 14.7 mg of one of sugars or amino acids and a freeze dry powder of cholesterol oxidase free of additive were compared as follows. The freeze dry powders were preserved at 37°C for one week, and the residual activity was measured. Moreover, after said freeze dry powders were preserved, they were dissolved in a 50 mM PIPES buffer (pH 7.0) so that said powders became 100 U/mL for an excess test, the solutions were preserved at 37°C, the formation of turbidity was

determined by OD_{660}, and the turbidity was confirmed by visual observation. The results are shown in Table 1 and Table 2.

[0023]

[Table 1]

Additive	Residual Activity (%)	OD_{660}	Visual Turbidity
None	60.5	1.368	x
BSA	88.1	1.0	x
Sucrose	73.3	0.488	Δ
Glucose	91.4	0.236	Δ
Trehalose	74.3	0.44	Δ
Lactose	81.8	0.384	Δ
Sodium glutaconate	70.5	0.316	Δ
Mannitol	40.3	0.46	Δ
Sodium glutamate	76.4	0.216	Δ
Glycine	66.0	1.08	Δ
			X

[0024]

[Table 2]

Combination with BSA			
Additive	Residual Activity (%)	OD_{660}	Visual Turbidity
Sucrose	88.6	0.136	o
Glucose	84.0	0.024	o
Trehalose	83.9	0.116	o
Lactose	84.4	0.168	o
Sodium glutaconate	87.7	0.096	o
Mannitol	67.9	0.116	o
Sodium glutamate	78.9	0.164	o
Glycine	101.1	0.076	o
			o

[0025] When the freeze dry powder of the stabilized cholesterol oxidase of present invention was preserved at 37°C, it was confirmed that a sufficient activity was maintained for one week and the formation of turbidity was also improved to a level of

nearly no problem in practice. On the other hand, when the freeze dry powder of the addition-free cholesterol oxidase was preserved at 37°C for one week, only 60% of activity was remained and no significant turbidity was found. For the freeze dry powder added with BSA alone, the residual activity was 88%, but a significant formation of turbidity was observed.

[0026] Actual Example 2

14.7 mg of lysine was added to and mixed with 1 mL of a cholesterol oxidase solution (2,000 U/mL), and then the mixture was freeze-dried to obtain 50 mg of a freeze dry powder. Similarly, 17.2 mg of BSA and 14.7 mg of lysine were added to and mixed with 1 mL of a cholesterol oxidase solution (2,000 U/mL), and then the mixture was freeze-dried to obtain 50 mg of a freeze dry powder. These freeze dry powders were preserved at 37°C for one week, and the residual activity was measured. After said freeze dry powders were preserved, they were dissolved in a 50 mM PIPES buffer (pH 7.0) so that said powders became 100 U/mL for an excess test, the solutions were preserved at 37°C, the formation of turbidity was determined by OD_{660}, and the turbidity was confirmed by visual observation. The result is shown in Table 3.

[0027]

[Table 3]

Additive	Residual Activity (%)	OD_{660}	Visual Turbidity
Lysine	102.2	0.072	○
Lysine + BSA	101.9	0.008	◎

[0028] Actual Example 3

21.2 mg of BSA (made by Organotechnic Co.), 16.8 mg of lysine (lysine 1) or 33.6 mg (lysine 2) were added to and mixed with 1 mL of a cholesterol oxidase solution (2,000 U/mL), and then the mixture was freeze-dried to obtain a freeze dry powder. The resultant freeze dry powders of cholesterol oxidase were preserved at 37°C for two weeks, and the residual activity was measured. After said freeze dry powders were preserved, they were dissolved in a 50 mM PIPES buffer (pH 7.0) so that said powders became 100 U/mL for an excess test, the solutions were preserved at 37°C, the formation of turbidity was determined by OD_{660}. The result is shown in Table 4.

[0029]

[Table 4]

Additive	Residual Activity (%)	OD_{660}	Visual Turbidity
Lysine 1	98.2	0.022	◎
Lysine 2	100	0.010	◎

[0030] When the cholesterol oxidase stabilized by the present invention was preserved at 37°C, it was confirmed that a

sufficient activity was maintained for two weeks and the formation of turbidity was also improved to a level of nearly no problem in practice.

[0031] Actual Example 4

18.6 mg of BSA and 14.8 mg of lysine were added to and mixed with 1 mL of a cholesterol oxidase solution (2,000 U/mL), and 18.6 mg of BSA, 14.8 mg of lysine and 14.8 mg of sucrose were further added, respectively, and then the mixtures were freeze-dried to obtain freeze dry powders.

[0032] The resultant freeze dry powders of cholesterol oxidase were preserved at 37°C for one week, and the residual activity was determined. After said freeze dry powders were preserved, they were dissolved in a 50 mM PIPES buffer (pH 7.0) so that said powders became 100 U/mL for an excess test, the solutions were preserved at 37°C, and the turbidity was determined by OD_{660}. The result is shown in Table 5.

[0033]

[Table 5]

Additive	Residual Activity (%)	OD_{660}	Turbidity
BSA + lysine	92.9	0.011	◎
BSA + sucrose	89.4	0.153	○
BSA + lysine + sucrose	98.3	0.074	○

[0034] When the cholesterol oxidase stabilized by the present invention was preserved at 37°C, it was confirmed that a

sufficient activity was maintained for one week and the formation of turbidity was also improved to a level of nearly no problem in practice.

[0035] Comparison Example 1

29 mg (0.5 M), 17.5 mg (0.3 M) and 8.8 mg (0.15 M) of sodium chloride were added to and mixed with 1 mL of a cholesterol oxidase solution (2,000 U/mL), respectively, and then the mixtures were freeze-dried to obtain freeze dry powders. The resultant freeze dry powders of cholesterol oxidase were preserved at 37°C for two weeks, and the residual activity was measured. After said freeze dry powders were preserved, they were dissolved in a 50 mM PIPES buffer (pH 7.0) so that said powders became 100 U/mL, the solutions were preserved at 37°C, and the formation of turbidity was visually observed. The result is shown in Table 6.

[0036]

[Table 6]

Additive	Residual Activity (%)	Turbidity
0.5 M NaCl	88.4	x
0.3 M NaCl	80.1	x
0.15 M NaCl	100	x

Thus, the formation of turbidity with the alkali metal is striking.

[0037]

[Effects of the Invention] A cholesterol oxidase formulation having excellent stabilization effect and turbidity can be obtained by adding lysine in the present invention. It has been known so far that bovine serum albumin, sugars and amino acids are used as stabilizers of various enzymes, but an even more excellent stabilization effect to heat is found by combining them than in case of using them separately. In the present invention, an effect of inhibiting the formation of turbidity by their combinations is very high, thus a stable enzyme formulation can be obtained. Accordingly, the present invention has an advantage that the enzyme formulation can be used as a biochemical reagent without pretreatment.